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A beads-based biofertilizer containing *Bacillus megaterium* for cabbage

T.M. Quynh[1*], N.T.T. Thuy[2], T.X. An[1], N.T. Thom[1], N.V. Binh[1], H.D. Sang[1], T.B. Diep[1], N.T. Ha[3], L.T.M. Luong[3]

[1]Hanoi Irradiation Center, Vietnam Atomic Energy Institute
[2]Faculty of Biology, University of Science, Vietnam National University, Hanoi
[3]Soils and Fertilizers Research Institute

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Abstract:

The aim of this study was to study the use of a beads-based biofertilizer in crop production. The study focuses on the production of a beads-based biofertilizer containing spores and vegetative cells of *Bacillus megaterium (B. megaterium)* VACC 118. It also evaluated the quality of the biofertilizer and its effects on the growth and yield of cabbage. The results show that the density of *B. megaterium* in the beads-based biofertilizer reached 4.34×10^{10} CFU/g and did not change after 6 months of storage. The degree of swelling of the beads was 1.57 times after 24 hours of immersion in an alkaline solution. The number of released cells of *B. megaterium* reached 4.2×10^{8} CFU/ml after 1 week of soaking in the alkaline solution. The application of the beads-based biofertilizer with NPK fertilizer increased the rate of folded plant, the fresh weight of head and the yield of cabbages grown in alluvial soil. When the beads-based biofertilizer was used along with a 20% reduction of the recommended NPK dosage, the yield of cabbage still increased by 12.36% compared to the control. This indicates that the beads-based biofertilizer can partially replace for chemical fertilizers.

Keywords: **beads-based biofertilizer, *B. megaterium*, cabbage growth and yield.**

Classification number: **3.1**

Introduction

Fertilization is essential for providing nutrients for the soil to facilitate plant growth and improve crop yields. Fertilizers in general and chemical fertilizer in particular can directly promote plant growth. According to the Food and Agriculture Organization (FAO), chemical fertilizers may be responsible for 55% of the increase in global yields of agricultural products and that each kilogram of NPK fertilizer applied to the crop can produce 10 kg to the yield [1]. However, long-term overuse of these inorganic fertilizers caused serious risks to our environment, affecting both food quality and eco-systems. Therefore, new safety-friendly fertilizers, such as biofertilizers and slow-release fertilizers, have been developed to partly replace inorganic fertilizers [2-5].

In Vietnam, probiotic and biofertilizers have been studied since the late 1980s, but only a few products have been developed by research universities and institutes [6]. Therefore, several imported biofertilizers have recently been approved for use in organic farming and sustainable agriculture. Most of the existing biofertilizers are carrier-based inoculants of nitrogen-fixing, phosphorous-solubilizing, or plant-growth-promoting bacteria. After fertilization, these beneficial microbes can easily colonize in the rhizosphere to deliver nutrients to plants, regulate phytohormones, and control phytopathogens. The microorganisms are encapsulated in carriers to protect them from adverse stress factors (such as pH, temperature, and radiation) during storage. Peat, clay, and bentonite are the most frequently used carriers due to their abundance, availability, and low cost. But, these materials can easily be contaminated due to their high nutrient content [7]. Therefore, polymeric-based carriers have been developed for the production of biofertilizersas these organic compounds can be utilized as a carbon source for bacteria and, therefore, to prolong their survival during storage [8].

* *Corresponding author: Email: tmqthuquynh@gmail.com*

In addition, the polymer molecules can crosslink to form three-dimensional structures that can protect the biofertilizers from contamination.

Alginate gels are widely used as polymer carriers due to their good biodegradability and compatibility. After inoculation, bacteria can incorporate to inthe gel in the wet or concentrated dry beads, which are easy to store, transport, and apply. However, these alginate beads are nonuniform, highly porous, andhave poor mechanical stability because alginate solution is very viscous and weak. Fillers such as starch may be added to the formulation to increase the dry matter in the beads, improving their mechanical resistance and allowing for a gradual release of beneficial microbes into the soil [9]. However, the insolubility and sedimentation of starch in the encapsulating solution can result in an unstable solution. Therefore, modified starch is often added to obtain a regularly dispersed solution for encapsulation, as reported by Ivanova, et al. [10]. Modified starch is easily obtained through irradiation, and this radiation method has proved a useful tool for the modification of natural polymers [11]. In our previous study, we found that modified starch with improved water solubility and dispersion was obtained by gamma irradiation at 3.5 kGy [12]. In addition, the modified cassava starch was also pasteurized during radiation treatment. Therefore, for this study, radiation-modified starch was mixed with sodium alginate to prepare a carrier for biofertilizer.

B. megaterium is a rod-like, gram-positive, plant-growth-promoting microorganism. These bacteria increase the availability of P and K in soil and produce indole acetic acid (IAA), a phytohormone that can improve plant growth. Their vegetative cells completely sporulate through heat inactivation, and the resulting spores, which are resistant to environmental stimuli, can easily germinate again under suitable conditions. Because the spores can survive for long periods in harsh conditions, they can inoculate onto the alginate-starch carriers for the production of biofertilizers that can be stored for longer.

In a recent study, *B. megaterium* VACC 118 strain, a plant-growth-promoting bacteria that can produce to 415 µg/ml of IAA after 5 days of cultivation, was selected from the culture collection of the Soils and Fertilizers Research Institute [13]. In order to produce an environmentally friendly fertilizer that can promote plant growth in unfavourable conditions, contribute to reducing pollution caused by agricultural chemicals and adapt to climate change, we attempted to produce alginate-starch bead carriers containing spores and vegetative cells of *B. megaterium* VACC 118.

Materials and methods

Materials

Cassava starch was purchased from Taiky Food, Vietnam. *B. megaterium* VACC 118 strain was selected from the culture collection of the Soils and Fertilizers Research Institute. Healthy seedlings of cabbage were kindly supplied by the Research Center for Fertilizers and Plant Nutrients at the Soils and Fertilizers Research Institute. All chemicals and reagents were industrial grade.

Methods

Preparation of the bead-based biofertilizer: B. megaterium cells were incubated in King's B for activation and then transferred to soybean extract medium and cultivated at 30^0C in favourable conditions for 5 days for sporulation. The liquid culture at the stationary phase was concentrated to a density of about $1.3\text{-}2.5\times10^{11}$ CFU/ml by centrifugation at 3,000 rpm and then collected for encapsulation.

The process for preparing the biofertilizer is presented in Fig. 1. Briefly, a modified starch was prepared from commercial cassava starch using gamma irradiation, as described in our previous study [12]. This was mixed with a pre-sterilized sodium alginate solution to form a starch-alginate (SA) solution of 2% sodium alginate and 33% modified starch. Then, the concentrated *B. megaterium* culture was homogenized with the SA solution at a volume ratio of 1:2 in a sealed bath and dropped onto a pre-sterilized solution of 2% $CaCl_2$ under magnetic stirrer for precipitation. The resulting beads were stabilized by further stirring for 30 minutes and were then washed with sterile water before drying. All manipulations were performed under aseptic conditions in clean laminar (Fig. 2).

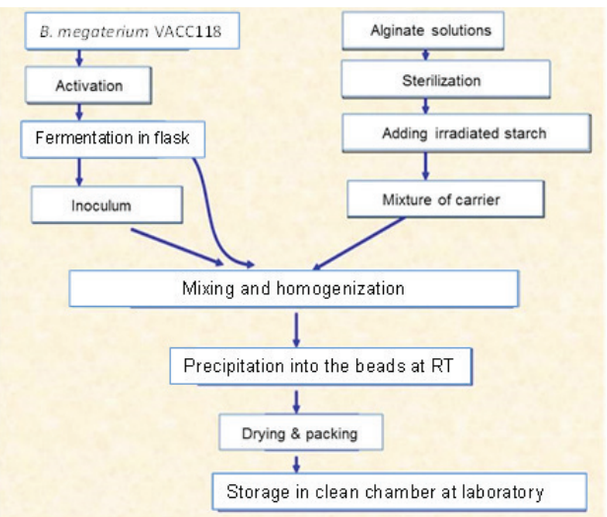

Fig. 1. Experimental process of preparation of bio-fertilizer.

Fig. 2. Production of bio-fertilizer beads in pilot scale.

Evaluation of the beads-based biofertilizer: the dried biofertilizer beads were immersed in a sterile saline solution (NaCl 0.85%, pH 7.0) for 24 hours at room temperature, and the swelling degree in percentage (PS) was measured by obtaining the weight ratio of swollen (W_s) to initial (W_d) beads using the following equation:

$$PS = 100\times(W_s - W_d)/W_d \quad (1)$$

In addition, 1 g of the dried beads wasimmersed in 100 ml of sterile saline for a week under weak stirring at room temperature for release test. The number of released cells in the saline solution was determined after 1, 2, and 7 days. For this experiment, the density of *B. megaterium* was determined by culturing the bacterial cells on a Luria Bertani (LB) agar plate supplemented with L-tryptophanin accordance with TCVN 10784:2015 [13].

The dried beads were packed in aluminium foil and placed in a plastic bag, and the packages were kept in a clean chamber. The effects of storage time on the viability of bacteria in the fertilizer were investigated after 7, 30, 90, and 180 days of storage at room temperature. First, 1 g of the beads were immersed in 10 ml of sterile saline for 30 minutes for hydration. The hydrated beads were moved to 100 ml of a 10% sodium tricitrate solution for a further 30 minutes, gently vortexed into suspension, and plated on LB agar. After incubation at 37^0C for 48 hours, the live cells in the beads were counted.

The effects of the beads-basedbiofertilizer on growth and yield of cabbage: together with NPK, the fertilizer beads were applied to cabbage once at a rate of 20 kg/ha before planting. The healthy seedlings of cabbage were planted in 24 m^2 testing plots containing alluvial soil at Thanh Da, Phuc Tho, Hanoi from January to March 2019. Each treatment was replicated three times. The experiment was arranged according to acompletely randomized block design, and the cabbage fertilized with 120 kg N, 60 kg P_2O_5 and 80 kg K_2O per ha as the control (DC) [14]. Treatments included TN1: NPK plus the beads-based biofertilizer and TN2: 80% NPK plus the beads-based biofertilizer. Plant growth was measured by recording plant height, rate of unfolded plant, head height and weight during the vegetative stage. The whole plot of cabbage heads was harvested, and the productivity of the cabbage and the yield increase were calculated [15].

Statistical analysis: the data were statistically analysed using Excel and IRRISTAT software.

Results and discussion

Quality of the beads-based biofertilizer

The dried beads containing *B. megaterium* VACC118 were prepared as a biofertilizer. The increased water solubility and reduced intermolecular hydrogen bonds of the radiation-modified starch helped the starch molecules to disperse well in the encapsulating solution [12]. Gamma irradiation also resulted in an increase in gelation of the modified starch. The results show that the beads-based biofertilizer has a relatively homogeneous shape and a higher dry matter content and that the *B. megaterium* cansurvive and grow better in the beads of alginate-modified starch.

Table 1. Quality of the beads-based biofertilizer.

Parameter	Measurement	Current regulation
Moisture (%)	9.4	≤30
pH	5.7	≥5
Density of useful cells (CFU/g)	4.34×10^{10}	$\geq10^8$
Salmonella (CFU/g)	Not detected	Negative
E. coli (MPN/g)	Not detected	1.1×10^3

Table 1 shows that the beads-based biofertilizer meets all the current requirements for biofertilizer and that the density of beneficial microbe in the beads-based biofertilizer at $4.34\text{x}10^{10}$ CFU/g, is much higher than the required density. This means that the products can be kept for longer. Moreover, the low moisture content of the product can also limit contamination, further prolonging its storage period. The density of *B. megaterium* in our biofertilizer is similar to the density of *A. brasilense* (10^{10}-10^{11} CFU/g) in the alginate-starch capsules produced by Ivanova, et al. [10] but higher than that ($8.6\text{x}10^8$ CFU/g) of the microbial inoculants produced by Thu Ha Nguyen, et al. [13]. The significant difference in the density of the beneficial microbes can be explained by the different encapsulating cultures.

Table 2. Rate of release of *B. megaterium* from the biofertilizer.

Soaking time (days)	Swelling degree (%)	Number of released cells (CFU/ml)
1	57.0	7.2×10^7
2	–	1.15×10^8
7	–	4.2×10^8

As shown in Table 2, the swelling degree of the beads-based biofertilizer reached 57%, which is equivalent to 1.57 times after 24 hours of immersion. Not only did the weight of the swollen beads increase, but their diameter also expanded after absorbing water, making it easier to releasing the cells from the beads. During the first day, the number of released cells was 7.2×10^7 CFU/ml. It was 1.15×10^8 CFU/ml in the second day and reached 4.2×10^8 CFU/ml after 1 week of soaking. The results suggest that the bacteria were not immobilized to the carrier but could easily move to the rhizosphere after inoculating to the soil. In fact, the release rate of surviving bacteria from the fertilizer to the soil is rather slow, because the dried beads have to be hydrated first.

Table 3. The survival of *B. megaterium* in the beads-based biofertilizer during the storage.

Time of storage (days)	Density of surviving cells (CFU/g)
0	4.34×10^{10}
7	4.26×10^{10}
30	4.18×10^{10}
90	3.75×10^{10}
180	2.31×10^{10}

The viability of the *B. megaterium* cells after 7, 30, 90, and 180 days storage is shown in Table 3. The results show that the density of *B. megaterium* in the beads-based biofertilizer does not appear to change during storage. The density of *B. megaterium* after 6 months storage was still high enough, which suggests that the fertilizer could be stored for longer. In a recent study, Thu Ha Nguyen, et al. [13] reported that the density of *B. megaterium* reduced to 2.6 from 8.6×10^8 CFU/g. However, the biofertilizer in their experiment contained four kinds of microbes mixed with cassava starch and rice bran as the carrier. The fact that various bacteria strains display antagonistic activities may have influenced the development of the microbial population in the inoculant.

According to Altuhaish, et al. [16], the density of surviving bacteria in biofertilizer was not significantly reduced after 3 months of storage. Thirumal, et al. [17] also found that the density of *B. megaterium* in an alginate-based carrier decreased slightly after longer storage periods but was about 7×10^8 CFU/g, meaning that their biofertilizer still in good conditions after 8 months of storage. This indicates that the alginate-based carriers used in the present study can prolong the storage of microbial fertilizers.

The effects of the beads-based biofertilizeron growth and yield of cabbage

Table 4. The effects of the beads-based biofertilizer on growth and yield of cabbage.

Formulation	Rate of folded Plan (%)	Fresh weight of head (kg)	Yield (tones/ha)	Increase (%)
DC	95.8	1.53	43.55	-
TN1	97.0	1.74	49.24	13.07
TN2	96.3	1.72	49.05	12.36
$LSD_{0.05}$			3.71	
CV			4.1	

DC: control (100% NPK as per local standard); TN1: 100% NPK + biofertilizer; TN2: 80% NPK + biofertilizer; $LSD_{0.05}$: least significant difference at 0.05; CV: coefficient of variation.

The effects of the beads-based biofertilizeron the growth and yield of cabbage planted in alluvial soil were determined and are presented in Table 4. The results reveal that the growth and yield of the cabbage inoculated with the beads-based biofertilizer together with NPK were better than the growth and yield of the cabbage treated with NPK only (DC). The application of the beads-based biofertilizer provided nutrients and phytohormones that stimulated plant growth, which resulted in a yield that was 13.07% greater than that of the control. When there commended NPK dosage was reduced by 20%, the yield of cabbage was still 12.36% greater than that of the control. Abou El Magd, et al. also reported a positive effect of biofertilizer on cabbage [18].

Conclusions

The biofertilizer formed from *B. megaterium* VACC118 and an admixture carrier of sodium alginate and radiation-modified cassava starch has a *B. Megaterium* density of $4.34x10^{10}$ CFU/g, which is much higher than the current requirement. The beads-based biofertilizer can easily absorb water and expand to release the living cells in the aqueous medium. The density of *B. Megaterium* did not decrease after 6 months of storage. The application of the beads-based biofertilizer with NPK increased the rate of folded plant, fresh weight of head, and yield of the cabbages grown on alluvial soil. When the beads-based biofertilizer used and the recommended NPK dosage was reduced by 20%, the yield of cabbage was still 12.36% greater than that of the control. This indicates that the beads-based biofertilizer can replace a proportion of chemical fertilizer.

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